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APPLICATION FOR LETTERS PATENT

for

PLANT STRESS-REGULATED GENES

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TITLE OF THE INVENTION

PLANT STRESS-REGULATED GENES

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application is a continuation of international application number PCT/EP02/01993, filed February 22, 2002, designating the United States of America, published in English February 13, 2003, corresponding to international publication number WO 03/012096 A2, the contents of which are incorporated herein by this reference in its entirety.

TECHNICAL FIELD

[0002] The present invention relates to a method to isolate plant genes or gene fragments that are regulated by stress, preferably oxidative stress. The method comprises isolation of plant material, adaptation of the plant material to stress, differential expression of genes or gene fragments in adapted and nonadapted plant material, and isolation of the differentially expressed genes or gene fragments. The invention further relates to the genes or gene fragments that can be obtained by this method and to the use of these genes or gene fragments to modulate plant stress tolerance.

BACKGROUND

[0003] Plant molecular responses to environmental stresses are generally very complex and often result in alteration of gene and protein expression, as well as in changes in metabolic profiles (Sandermann *et al.*, 1998; Jansen *et al.*, 1998; Somssich and Hahlbrock, 1998; Bartels *et al.*, 1996). At least some of those stress responses are required for enhanced stress tolerance as the moderate doses of many stresses increase plant resistance to deleterious stress conditions. For example, raising the temperatures slowly to high, nonlethal temperatures allows plants to tolerate temperatures that are normally lethal, a phenomenon referred to as acclimation (Vierling, 1991). Similarly, exposing maize plants to 14°C acclimates them to lower temperatures that would normally cause chilling injuries (Prasad *et al.*, 1994). Also, pathogen infection often leads to resistance against subsequent challenges by the same or even unrelated pathogens (reviewed in Sticher *et al.*, 1997). This phenomenon of induced stress tolerance is not restricted to the same kind

of stress and cross-tolerance induced by different kinds of stresses have been reported (Örvar *et al.*, 1997; Orzech and Burke, 1988; Keller and Steffen, 1995; Cloutier and Andrews, 1984).

[0004] Much of the damage due to environmental constrains has been attributed to the excess production of active oxygen species (AOS), so-called oxidative stress (reviewed in Inzé and Van Montagu, 1995). Plant cells acclimated to heat and cold, as well as plants expressing systemic acquired resistance to pathogens, also show enhanced capacity to tolerate oxidative stress (Banzet *et al.*, 1998; Seppänen *et al.*, 1998; Strobel and Kuc, 1995). This suggests that induced tolerance to oxidative stress is part of the adaptation mechanism to environmental stresses and likely contributes to the observed phenomenon of cross-tolerance. However, little is known in plants about molecular mechanisms underlying induced tolerance to oxidative stress.

[0005] In contrast, adaptive responses to various oxidants have been extensively studied in bacteria and yeast. In both *E. coli* and *S. cerevisiae,* adaptation to oxidative stress is an active process requiring *de novo* protein synthesis (Davies *et al.*, 1995; Storz *et al.*, 1990). At least 80 proteins are induced by adaptive amounts of oxidants in *E. coli*; 40 of them belong to H_2O_2 stimulon and 40 to $O_2^{\bullet-}$ stimulon. Among the induced enzymes are antioxidant enzymes, DNA repair enzymes, heat shock proteins, and glucose-6-phosphate dehydrogenase implicated in energy homeostasis (reviewed in Demple, 1991).

[0006] Yeast, similarly to bacteria, possess at least two distinct but overlapping adaptive stress responses to oxidants: one induced by H_2O_2 and the other by $O_2^{\bullet-}$ generating compounds (Jamieson, 1992). The H_2O_2 stimulon has been analyzed by comparative two-dimensional gel analysis of total cell proteins isolated after treatment with low doses of H_2O_2 (Godon *et al.*, 1998). Such a treatment resulted in synthesis of at least 115 proteins and repression of 52 proteins. 70% of those proteins have been identified and classified into cellular processes such as antioxidant defenses, heat shock responses and chaperone activities, protein turnover, sulphur, amino acids, purine, and carbohydrate metabolism. Notably, carbohydrate metabolism was redirected to the regeneration of NADPH, which provides reducing power necessary for the detoxification of active oxygen species.

[0007] In plants, tolerance to oxidative stress has been previously associated with enhanced activity of antioxidant enzymes and levels of antioxidant metabolites (reviewed in Inzé and Van Montagu, 1995). In addition, Banzet *et al.* (1998) have demonstrated that other stress

proteins are likely implicated in acquisition of oxidative stress tolerance by plant cells, similarly as in lower organisms. Expression of small heat shock proteins correlated with adaptation of tomato cells to oxidative stress and, additionally, heat shock pretreatment was able to enhance resistance of those cells to oxidative stress. However, no comparative genome-wide characterization of induced adaptive responses to oxidative stress has been undertaken in plants.

SUMMARY OF THE INVENTION

[0008] A genomic approach was used to study the adaptive responses to oxidative stress in leaf tissue of *Nicotiana tabacum.* The redox-cycling compound, methyl viologen (MV; paraquat), was used to induce an adaptive response to oxidative stress, as AOS signaling may be important during the defense against both biotic and abiotic stresses in plants (Levine *et al.*, 1994; Prasad *et al.*, 1994; Banzet *et al.*, 1998; Chamnongpol *et al.*, 1998; Alvarez *et al.*, 1998; Karpinski, 1999). Surprisingly, we found that adaptive amounts of MV enhance the tolerance of tobacco leaf tissues to oxidative stress imposed by toxic levels of the same oxidant. Moreover, adaptation to oxidative stress is associated with induction/repression of approximately 170 genes, and partial characterization of induced genes shows that they are implicated in distinct cellular processes. Several of these defense responses induced by adaptive amounts of oxidants have so far never been associated with the antioxidant response.

[0009] It is a first aspect of the invention to provide a method to isolate stress-regulated genes or gene fragments, the method comprising:

(a) isolating plant material;

(b) inducing stress adaptation in plant material;

(c) checking differential expression between stress-adapted and nonadapted plant material; and

(d) isolating differentially expressed genes or gene fragments.

[0010] Plant material can be any plant material, such as parts of, or complete, roots, stems or leaves. Plant material may include more than one plant tissue, up to a complete plant. Preferably, the plant is a tobacco plant. Even more preferable, the plant material is leaf material.

[0011] Induction of stress adaptation is preferentially carried out by applying sublethal stress to the plant material. Stress can be any biotic or abiotic stress, such as fungal or bacterial

infection, heat or cold treatment, or oxidative stress. Preferably, the stress is oxidative stress. More preferably, the oxidative stress is applied by putting the plant material in a solution comprising an adequate amount of methyl viologen (methyl viologen pretreatment). Alternatively, the sublethal stress phase may be followed by a period of stronger stress. The stronger stress may even result in significant cell damage when applied to unadapted plant material.

[0012] Differential expression includes induction as well as repression. Checking differential expression can be done with all the differential expression or differential display techniques known to the person skilled in the art, such as, but not limited too, messenger substraction, filter hybridization or micro-array techniques.

[0013] Isolation of the differentially expressed genes may be direct or indirect, i.e., by direct isolation of the differentially expressed nucleic acid such as mRNA or cDNA, or by isolation of the genes from a library, on the basis of the results identifying the gene, such as filter hybridization or micro-array. Preferably, the differentially expressed genes or gene fragments are isolated using PCR-based techniques.

[0014] A further aspect of the invention is a gene, or gene fragment, obtained by the method according to the invention. A preferred embodiment is a gene or gene fragment, comprising a sequence selected from any of the sequences from SEQ ID NO:1 to SEQ ID NO:167.

[0015] Clone names of these sequences, their expression pattern and an indication of the function by homology search is summarized in Table 1.

[0016] In one embodiment, a gene encoding a protein comprising, or preferably essentially consisting of, or more preferably consisting of, SEQ ID NO:169. Preferably, the gene comprises SEQ ID NO:168. More preferably, the gene is essentially consisting of SEQ ID NO:168 and, even more preferably, the gene is consisting of SEQ ID NO:168.

[0017] Still another aspect of the invention is the use of a gene or a gene fragment according to the invention, or a gene that is at least 60% identical, preferably 80% identical, and more preferably 90% identical to the gene or gene fragment according to the invention, or a gene fragment from a gene that is at least 60% identical, preferably 80% identical, or more preferably 90% identical to the gene or gene fragment according to the invention to modulate plant stress tolerance. A preferred embodiment is the use of a gene or gene fragment comprising SEQ ID

NO:168, preferably essentially consisting of SEQ ID NO:168, or more preferably consisting of SEQ ID NO:168. Preferably, the stress is oxidative stress. Preferably, the plant is tobacco.

[0018] A special embodiment is the use of a gene fragment according to the invention, whereby the gene fragment is a promoter. Although the gene fragments isolated by the differential expression procedure may be coding sequences that do not comprise the promoter of the gene, a person skilled in the art can isolate the promoter of a gene when the coding sequence is known. As a nonlimiting example, the coding sequence can be used as a probe against a genomic library, whereby the positive scoring clones are subcloned, and the positive subclone is sequenced. On the base of the sequence, the promoter part and the coding part, including the intron–exon boundaries, can be predicted using computer software, such as Genemark, Genscan or Grail. Alternatively, the full-length messenger RNA can be isolated and, on the base of its sequence, the start of transcription can be defined and the promoter can be localized.

[0019] Another aspect of the invention is a vector comprising a gene or a gene fragment according to the invention. The vector may be any vector suitable for eukaryotic cells, as is known to the person skilled in the art, and includes, but is not limited to, self-replicating vectors, integrative vectors and virus-based vectors. Preferably, the vector is a plant transformation vector and the eukaryotic cell is a plant cell.

[0020] Still another aspect of the invention is a method to modulate stress tolerance in a plant cell or plant comprising the introduction of the vector according to the invention in the plant cell or plant. Introduction of the vector in the plant cell or plant can be realized by any suitable technique known to the person skilled in the art and includes, but is not limited to, transformation techniques such as electroporation, particle bombardment or *Agrobacterium*-mediated transformation, floral dip transformation or sexual techniques such as crossing.

[0021] A further aspect of the invention is a plant cell or plant, comprising a vector according to the invention. Preferably, the plant cell or plant is a tobacco plant cell or plant.

BRIEF DESCRIPTION OF THE DRAWINGS

[0022] FIG. 1. Effect of different concentrations of methyl viologen on leaf disc damage. Three leaf discs were floated on solution with assigned methyl viologen concentrations for indicated time periods. Ion leakage was measured as conductivity of the medium at indicated time intervals.

The experiment was done in duplicate and the presented value is the average of both measurements. The conductivity of the solution was subtracted from the measured values.

[0023] FIG. 2. Effect of MV pretreatment on leaf disc tolerance to 1 μM methyl viologen. Leaf discs that were pretreated for 17 hours with water (grey bars) or 0.1 μM methyl viologen (black bars) were exposed to 1 μM solution of methyl viologen. Ion leakage was measured as conductivity of the medium in the course of the treatment at regular intervals. The conductivity of the solution was subtracted from measured values. Presented values are average values of nine independent experiments.

[0024] FIG. 3. Expression of *GPx* and *SodCc* during the treatment with 1 μM methyl viologen. Leaf discs pretreated with water (0) or 0.1 μM MV (0.1) for 17 hours were exposed to 1 μM methyl viologen and expression of a glutathione peroxidase gene (*GPx*) and a gene encoding cytosolic CuZnSOD (*SODCc*) was analyzed. Total RNA (5 μg) was extracted from six leaf discs sampled in two independent experiments at indicated times and subjected to Northern analysis. The same membrane was used for hybridization with both genes. Hybridization of the constitutive actin gene was used as a loading control (bottom panel).

[0025] FIG. 4. Expression of genes isolated by differential display during the pretreatment with 0.1 μM methyl viologen and the treatment with 1 μM methyl viologen. Total RNA was extracted from nine leaf discs sampled at indicated times before (c) and during the pretreatment with 0.1 μM MV (0.1) or water (0), and after exposure of pretreated samples to 1 μM MV. Blots with 15 μg total RNA each were prepared in quadruplicate and checked for equal loading by methylene blue staining. Each membrane was reused several times.

[0026] FIGS. 5A and 5B. Resistance to MV of *A. thaliana* transformed with WRKY11 fused to the VP16 activation domain, under control of the 35S promoter. (A) The control plate without MV; (B) the test plate with 2 μM MV. WV9 and WV4 are transformed lines; C24 is an untransformed control.

DETAILED DESCRIPTION OF THE INVENTION

[0027] While this invention is described in certain embodiments and by way of certain examples, the present invention can be further modified within the spirit and scope of this disclosure. This application is therefore intended to cover any variations, uses, or adaptations of the

invention using its general principles. Further, this application is intended to cover such departures from the present disclosure as come within known or customary practice in the art to which this invention pertains and which fall within the limits of the appended claims.

Definitions

[0028] As used herein "*Plant material*" can be any plant tissue such as root, stem or leaf. It may be a part of the plant, such as a disc excised from the leaf, up to the intact plant.

[0029] As used herein "*Adaptation*" means the application of a stress to the plant for a certain time, whereby the time and/or the level of stress are controlled in such a way that the stress applied over the time used is sublethal.

[0030] As used herein "*Sublethal stress*" refers to stress that may result in a specific gene expression pattern but is not leading to a lethal amount of cell damage. Detrimental tissue damage can be evaluated by several methods known to the person skilled in the art, but is preferably evaluated by measuring an increase in conductivity as described in the examples. An increase in conductivity in the stress situation, compared with a nonstressed reference situation by less than a factor 5, preferably less than a factor 2, as measured after 42 hours of stress application is considered as insignificant.

[0031] The term "*gene*," as used herein, refers to a polymeric form of nucleotides of any length, either ribonucleotides or deoxyribonucleotides. This term refers only to the primary structure of the molecule. The term includes double- and single-stranded DNA and RNA. It also includes known types of modifications, for example, methylation, "caps" substitution of one or more of the naturally occurring nucleotides with an analogue. It includes, but is not limited to, the coding sequence. It does include the regulatory sequences such as the promoter and terminator sequences.

[0032] As used herein "*Gene fragment*" may be any gene fragment of at least 40 contiguous nucleotides, preferably 60 nucleotides, more preferably 100 nucleotides, either coding or noncoding. A special case of gene fragment is the promoter of the gene.

[0033] As used herein "*Modulation of stress tolerance*" comprises both the increase of stress tolerance, as well as the decrease of stress tolerance, independent of the level of decrease or increase.

[0034] As used herein "*% identical*" is the percentage identity between two or more nucleic acid or amino acid sequences as measured by a TBLASTN search (Altschull *et al.*, 1997).

Plant Material and Cultivation Conditions

[0035] *Nicotiana tabacum* cv. Petit Havana SR1 plants were grown in a controlled environment chamber (Weiss Technik, Lindenstruth, DE) under 100 $\mu mol/m^2/s$ light intensity (photosynthetically active radiation), 16 hours light/8 hours dark regime, relative humidity of 70% and constant temperature of 24°C. The most expanded leaves (11-12 cm long x 7-8 cm wide) from five week old plants were used for experiments with methyl viologen.

Methyl Viologen Treatment

[0036] Leaf discs (1 cm in diameter) were punched with a cork-bore from the interveinal part of the leaf. Three leaf discs, each originating from different plants, were floated with the abaxial side up on 12 ml of methyl viologen solution in nanopure water or water solely in the case of control. Treatments were performed in controlled environment chambers, under the same conditions as for growth, except as otherwise indicated. Leaf discs for RNA extraction were drained on paper, rapidly frozen in liquid nitrogen and stored at -70°C. Ion leakage from the leaf discs was measured as conductivity of the solution using a conductivity meter (Consort, Turnhout, BE).

RNA Extraction and Northern Analysis

[0037] Total RNA was extracted from frozen leaf discs using TRIzol™ Reagent (Life Technologies, Paisley, UK) according to the manufacturer's instructions. RNA samples were treated prior to electrophoresis and resolved on 1% agarose gel as described by Shaul *et al.* (1996). The RNA was blotted on nylon membrane (Hybond-N, Amersham International plc., Buckinghamshire, UK or Qiabrane, Qiagen GmbH, Hilden, DE) by capillary blotting (Maniatis *et al.*, 1982). RNA was fixed to the membrane by cross-linking at 150 mJ/cm^2. To check the quality of RNA prior to hybridization, membranes were incubated for 15 minutes in 5% acetic acid and stained for five minutes in 0.04% methylene blue in 0.5 M sodium acetate (pH 5.2), and rinsed with water. After the staining and quality check, membranes were destained in 0.1 x SSC (Maniatis *et*

al., 1982) containing 0.5% SDS (w/v). Membranes were hybridized at 65°C in 50% formamide, 5 x SSC, 0.5% SDS and 10% dextran sulphate. ^{32}P-labeled RNA probes corresponding to the cDNA fragments of *GPx* (Criqui *et al.*, 1992), *SodCc* (pSOD 3-5′ fragment; Tsang *et al.*, 1991), *SodB* (pSOD 2-5′ fragment; Tsang *et al.*, 1991), *Cat1* (pCat1A; Willekens *et al.*, 1994), and *N. tabacum* actin (pRVA12; Aventis Crop Science, BE) were generated by the Riboprobe® System (Promega Corp., Madison, WI, USA). RNA probes corresponding to cDNA fragments isolated by differential display and cloned into pGEM®-T vector (Promega Corp., Madison, WI, USA) were generated according to the same protocol. Membranes were washed at 65°C for 15 minutes each in 3 x SSC (Maniatis *et al.*, 1982), 1 x SSC and 0.1 x SSC (stringent washing) containing 0.5% SDS (w/v). Membranes were exposed to the Storage Phosphor Screen and scanned with the PhosphorImager 445 SI (Molecular Dynamics Inc., Sunnyvale, CA, USA). Membranes were reused after stripping of the probe in 0.1 x SSC at 85°C. Removal of the probe was checked by autoradiography.

Differential display

[0038] Total RNA was treated with DNaseI prior to RT-PCR according to the manufacturer's instructions (Life Technologies, Paisley, UK). Alternatively, up to 20 µg of total RNA was incubated with 5 U DNaseI, 18 U Recombinant Ribonuclease Inhibitor (Promega Corp., Madison, WI, USA), 1 mM DTT in 80 µl of 10 mM Tris-Cl, pH 8.3, 50 mMKCl and 1.5 mM $MgCl_2$ for 30 minutes at 37°C. RNA was extracted with phenol/$CHCl_3$ (3:1), ethanol precipitated and dissolved in diethyl pyrocarbonate-treated water. mRNA differential display was performed with the RNA map™ kit (Gene Hunter Corp., Nashville, TN, USA), AmliTaq DNA polymerase (Perkin-Elmer, Branchburg, New Jersey, USA) and [^{33}P] dATP (0.2 µl/20 µl PCR reaction of 111 000 GBq/mmol; Isotopchim, Ganagobie-Peyruis, FR). 3.5 µl of each PCR reaction was mixed with 2 µl of loading dye and denatured at 95°C for five minutes prior to loading onto 6% DNA sequencing gel. Gels were electrophoresed at 90 Watts constant power until the xylene dye reached the bottom and dried at 80°C for about one hour. All the 20 decamers were used in combination with the four T_{12}MN primers provided with the kit. Bands with a differential expression pattern and larger than 200 bp were purified from the polyacrylamide gels and reamplified according to the instructions provided in the manual of the RNA map™ kit. Reamplified cDNA was ethanol precipitated and cloned into pGEM®-T vector (Promega Corp., Madison, WI, USA). Each clone

was assigned a number corresponding to the primer used, position on the gel and number of cDNA fragment within the isolated band (e.g., t 18-2-5 was amplified with primers $T_{12}MT$ and AP18, isolated as a second from the top of the gel, and after the cloning, the fifth colony was sequenced).

DNA sequence analysis

[0039] Three to six cDNAs originating from a single band were sequenced by primer walking using ABI Prism® BigDye™ terminator cycle sequencing kit (PE Applied Biosystems, Foster City, CA, USA). DNA sequence data were analyzed using the Wisconsin Package Version 9.1 (Genetics Computer Group (GCG), Madison, WI). The nucleotide sequences of all cloned cDNAs were compared with sequences deposited in GenBank, EMBL, DDBJ, and PDB databases, and translated DNA sequences were compared with protein sequences of GenBank CDS translations, PDB, SwissProt, PIR and PRF databases using the BLAST algorithm (Altschul *et al.*, 1997). The scoring matrix used by blastp search was BLOSUM62 (Henikoff and Henikoff, 1992). Gene homologues in the database were considered to be significant when the e-value was $<10^{-3}$ and the high-scoring segment pair identity was at least 20% for an amino acid sequence and 50% for a nucleotide sequence.

Plasmid construction

[0040] pWRKY11: WRKY11 cDNA was amplified from a cDNA library with primers EVVRA 28 and EVVRA 29 and cloned into pGEM-t™(Promega) *Pst*I and *Not*I sites via an intermediate cloning in the pZErO™ vector (Invitrogen).

[0041] pWRKY-pGSJ780A: The *Bgl*II-digested *WRKY11* sequence was cloned into the *Bam*HI site of the pGSJ780 binary vector (Bowler *et al.*, 1991).

[0042] pWRKY-VP16-pGSJ780A: The VP16 activation domain was amplified from pTETVP16 by primers EVVRA 26 and EVVRA30 and cloned as an *Xho*1 fragment into the *Xho*1 site of pWRKY11.

[0043] The WRKY-VP16 fusion was then cloned as a *Bgl*II fragment into the *Bam*HI site of pGSJ780A.

Arabidopsis transformation

[0044] Arabidopsis transformation was carried out by the floral dip method (Clouch and Bent, 1998). Selection of primary transgenics and progeny was based on transgene expression levels as determined by Northern blot analysis.

Stress assessment

[0045] Eighty plants of an F_2-progeny of the transgenic line WV4 (construct pWRKY-VP16-pGSJ780A) were grown on MS+Kanamycine for two and a half weeks. Fifteen kanamycine-resistant seedlings were transferred to plates containing ½ MS, 1% sucrose and 2 μM methyl viologen (paraquat) or to plates containing ½ MS, 1% sucrose for the controls.

[0046] Wild-type *Arabidopsis* plants were treated in a similar way (except for selection on Kanamycine).

[0047] Performance of plants was followed and pictures were taken after approximately three weeks.

Example I: Sensitivity of tobacco to methyl viologen

[0048] As a first step in studying adaptive responses to oxidative stress in tobacco, we wanted to establish an experimental system in which low doses of oxidant would induce adaptation to higher doses of the same compound. MV, a redox-active compound that enhances superoxide radical ($O_2^{\bullet -}$) formation mainly in chloroplasts, was used as an oxidant. In order to determine MV concentrations suited for the induction of adaptation and for the subsequent oxidative stress treatment, sensitivity of tobacco to MV was first determined. Leaf discs were floated on solutions with different concentrations of MV and ion leakage was monitored by measuring the solute conductance. If not scavenged, superoxide generated by MV is converted through redox-reactions into other active oxygen species (AOS) such as hydroxyl radicals that interact with biological molecules and cause oxidative damage (Halliwell and Gutteridge, 1989). Peroxidation of membrane lipids results in loss of membrane integrity that is reflected by enhanced cellular ion leakage. Concentrations lower than 0.2 μM MV caused very little increase in ion leakage from the leaf discs in comparison with water-treated controls and no visible damage was seen even after 42 hours of incubation (FIG. 1). These concentrations thus seemed most suitable for inducing adaptation to MV. When leaf discs were incubated in MV solutions at concentrations ranging from

0.2–2 μM MV, leaf damage measured as solute conductance clearly correlated with the applied dose of MV. This correlation was more or less linear within this range, suggesting that these doses of MV are most suited for monitoring differences in MV sensitivity between pretreated and control samples.

Example II: MV pretreatment induces tolerance and activates expression of antioxidant genes

[0049] To test whether exposure to sublethal amounts of MV enhances tolerance to higher, normally toxic amounts of this compound, tobacco leaf discs were floated on solutions with less than 0.2 μM MV and subsequently transferred to solutions within the molar range of 0.2-2 μM. Increase in tolerance was assessed by measuring the solute conductance. Leaf discs pretreated with water were taken as nonadapted controls. Protection against MV was most pronounced (40% in the mean compared to water-pretreated control samples) when leaf discs were pretreated with 0.1 μM MV for 17 hours (including eight hours dark period; referred to as "pretreatment") and subsequently treated with 1 μM MV for 11 hours (referred to as "treatment") (FIG. 2). These parameters for the pretreatment and the treatment were then used in all further experiments, unless otherwise stated. The specific conditions required for inducing adaptation were not investigated; yet, it was noticed that both the MV concentration and duration of the pretreatment were factors that affected the level of protection.

[0050] mRNA levels of several antioxidant genes were tested by Northern analysis during the pretreatment and the treatment. Both water and MV caused a rapid induction (one hour) of a glutathione peroxidase gene (*Gpx)* and a gene encoding cytosolic CuZnSOD (*SodCc*) (data not shown). *Gpx* and *SodCc* were only transiently induced in water-pretreated samples, suggesting that this induction was caused by the tissue wounding during leaf disc preparation. In contrast, pretreatment with 0.1 μM MV gave a persistent increase in *Gpx* and *SodCc* mRNA. After transfer to 1 μM MV, *Gpx* and *SodCc* were again induced in both water and MV-pretreated samples. However, the amount of both messengers remained consistently higher in MV-pretreated samples (FIG. 3). The above data indicate that induced tolerance is not just a physiological response but that it involves changes in nuclear gene expression and that *GPx* and cytosolic CuZnSOD are playing a role in the observed enhanced tolerance of pretreated samples.

Cat1 and *SodB* genes were also induced following the pretreatment, but their transcript levels declined during the subsequent treatment with 1 μM MV and no correlation could be established between their mRNA levels and enhanced tolerance.

Example III: Expression of a large number of genes implicated in distinct cellular processes is modulated by MV pretreatment

[0051] In order to identify which genes other than those encoding antioxidant enzymes would show altered mRNA levels during oxidative stress adaptation, reference samples placed in water for 17 hours, or samples pretreated with 0.1 μM MV for 17 hours (adapted leaf discs), were compared by differential mRNA display. To increase the fidelity of the differential display results, mRNA from two independent experiments was used to prepare cDNA, and reverse transcription was performed in duplicate for each RNA sample. Amplified cDNA from two separate experiments and two independent reverse transcription reactions were displayed next to each other on the sequencing gel. Eighty primer combinations yielded 243 bands larger than 150 bp that consistently showed differential expression between adapted and nonadapted samples. Two hundred two of them were up-regulated and 41 were down-regulated. Reamplified cDNA fragments larger than 200 bp were cloned and three to six cDNAs from 60% of all bands sequenced. Sequencing data revealed that 50% of sequenced bands contained two or more cDNA species and 30% of the bands were redundant. Taking into account this redundancy and assuming that only one cDNA species per band contributed to the differential expression pattern, the total number of genes with altered expression after MV pretreatment is estimated to be 170. Expression of 16 genes was further analyzed by Northern analysis with RNA from an independent experiment. The induction of 12 genes was confirmed, while four genes remained uninduced. Three out of these four genes were isolated from bands consisting of mixed cDNAs, indicating that they were not responsible for the differential expression pattern. The fact that expression of most of the isolated genes was reconfirmed by Northern analysis is a good indication of procedure fidelity and suggests that the number of genes transcriptionally responding to MV is close to the number estimated by sequencing data.

[0052] The nucleotide sequences and translations of 167 cDNAs isolated from differentially expressed bands were compared with nonredundant databases. Only 12 cDNAs were identical or highly similar (>90% over the whole sequence) to previously isolated tobacco genes.

Of the other 145 cDNAs, 36 were significantly similar to genes/proteins with known or predicted function, and 16 to genes with no assigned function. The high percentage of cDNAs (62%) for which no similarity was found in the database can, in part, be attributed to the fact that the isolated cDNAs mostly contain the 3′ untranslated region where sequence divergence is very high. The homologues of isolated cDNAs, of which the expression was tested and reconfirmed by Northern analysis, are listed in Table 2. Data shows that in addition to antioxidant genes, genes encoding chaperones (*DNAJ*), transporter proteins (*MDR*), dioxygenases (*DIOX*), enzymes of carbohydrate (*ATPC-L*), lipid (*Lox2, MFP*) and terpenoid metabolism (*EAS, VS*), regulatory proteins (*WRKY11, TPK*) and pathogen-related proteins (*PRB1b, CBP20*) are activated during MV-induced adaptation to oxidative stress in tobacco. The large number, as well as the functional diversity of genes transcriptionally responding to MV pretreatment, indicates that AOS activate a wide range of responses within the plant cells.

[0053] **Table 1:** list of stress-related genes with identification on the base of homology

Clone number	DD+/-	N+/-/=	homology E<10-3 with at least 20%amino acids or 50% nucleic acids identical nonredundant DNA and protein sequence databases (blastx/blastn)
a1-1-14.seq	+		SEQ ID NO:1
a1-1-7.seq	+		SEQ ID NO:166
a10-2-12.seq	+		hypothetical protein [Arabidopsis thaliana] (gb|AAD08932) SEQ ID NO:2
a10-4-1.seq	+		metallothionein-like protein type 2 Nicotiana plumbaginifolia (gb|U35225) SEQ ID NO:3
a10-4-12.seq	+		SEQ ID NO:4
a10-4-15.seq	+		SEQ ID NO:5
a14-1-1.seq	+	=	serine carboxypeptidase-like protein Oryza sativa (dbj|BAA04511) SEQ ID NO:6
a14-1-3.seq	+		SEQ ID NO:7
a14-1-4.seq	+		SEQ ID NO:8
a18-1-5.seq	+		EREBP-1 Matricaria chamomilla (dbj|BAA87068) SEQ ID NO:9
a18-1-8.seq	+		SEQ ID NO:10
a18-3-2.seq	+		SEQ ID NO:11
a18-3-3.seq	+		EIF-5A (eukaryotic initiation factor 5A2) Solanum tuberosum (sp|P56333) SEQ ID NO:12
a18-4-6.seq	+		SEQ ID NO:13
a19-3-1.seq	+		SEQ ID NO:14
a19-3-3.seq	+		SEQ ID NO:15
a19-3-4.seq	+		SEQ ID NO:16
a19-3-9.seq	+		SEQ ID NO:17

Clone number	DD+/-	N+/-/=	homology E<10-3 with at least 20%amino acids or 50% nucleic acids identical nonredundant DNA and protein sequence databases (blastx/blastn)
a20-1-3.seq	+		SEQ ID NO:18
a3-2-2.seq	-		ribosomal protein L12 (60S) Prunus armeniaca (sp|O50003) SEQ ID NO:19
a8-1-1.seq	-		SEQ ID NO:20
a8-1-2.seq	-		geranyl-geranyl reductase chlP-gene *Nicotiana tabacum* (emb|CAA07683) SEQ ID NO:21
a8-1-4.seq	-		early wound inducive gene *Nicotiana tabacum* (dbj|BAA95791) SEQ ID NO:22
a9-1-2.seq	+		epoxide hydrolase *Nicotiana tabacum* (gb|AAB02006) SEQ ID NO:23
a9-3-4.seq	+		immediate-early salicylate-induced glucosyltransferase (IS10a) *Nicotiana tabacum* (gb|U32643) SEQ ID NO:24
a9-4-1.seq	+		SEQ ID NO:25
a9-5-9.seq	+		SEQ ID NO:26
a9-6-11.seq	-		SEQ ID NO:27
a9-7-1.seq	+		SEQ ID NO:28
a9-7-10.seq	+		lipoxygenase LOX1 *Nicotiana tabacum* (emb|X84040) SEQ ID NO:29
a9-7-11.seq	+		SEQ ID NO:30
c1-1-3.seq	+		SEQ ID NO:31
c1-1-5.seq	+		SEQ ID NO:32
c1-2-2.seq	+		SEQ ID NO:33
c1-3-12.seq	-		SEQ ID NO:34
c10-3-1.seq	-		SEQ ID NO:35
c10-3-5.seq	-		SEQ ID NO:36

Clone number	DD+/-	N+/-/=	homology E<10-3 with at least 20%amino acids or 50% nucleic acids identical nonredundant DNA and protein sequence databases (blastx/blastn)
c11-2-1.seq	+		SEQ ID NO:37
c11-3-1.seq	+		SEQ ID NO:38
c11-3-3.seq	+		caffeoyl-CoA O-methyltransferase *Nicotiana tabacum* (emb|Z56282) SEQ ID NO:39
c13-1-6.seq	+		SEQ ID NO:40
c13-2-1.seq	+		L19 ribosomal protein *Nicotiana tabacum* (emb|Z31720) SEQ ID NO:41
c13-3-13.seq	+		23S 4.5S rRNA genes chlP-genes *Nicotiana tabacum* (gb|J01446) SEQ ID NO:42
c13-3-6.seq	+		SEQ ID NO:43
c14-1-60.seq	+		glycolate oxidase *Lycopersicon esculentum* (pir|T07032) SEQ ID NO:44
c14-2-10.seq	+		SEQ ID NO:45
c14-2-15.seq	+		ribosomal protein L35-like (60S) *Arabidopsis thaliana* (emb|CAB85998) SEQ ID NO:46
c14-3-4.seq	+		ribosomal protein L23a-like (60S) *Arabidopsis thaliana* (emb|CAB75762) SEQ ID NO:47
c14-5-1.seq	-		predicted protein *Oryza sativa* (dbj|BAA83350) SEQ ID NO:48
c14-6-11.seq	+		predicted protein *Arabidopsis thaliana* (pir|T02387) SEQ ID NO:49
c14-7-4.seq	+		SEQ ID NO:50
c15-1-2.seq	+		SEQ ID NO:51
c15-1-4.seq	+	+	pathogen- and wound-inducible antifungal protein CBP20 precursor *Nicotiana tabacum* (gb|AAB29959) SEQ ID NO:52
c15-11-2.seq	+		SEQ ID NO:53
c15-11-4.seq	+		SEQ ID NO:54
c15-2-8.seq	+		hypothetical protein *Arabidopsis thaliana* (emb|CAB88533) SEQ ID NO:55

Clone number	DD+/-	N+/-/=	homology E<10-3 with at least 20%amino acids or 50% nucleic acids identical nonredundant DNA and protein sequence databases (blastx/blastn)
c15-3-4.seq	+		hypothetical protein *Arabidopsis thaliana* (gb|AAF63779) SEQ ID NO:56
c15-6-2.seq	+		SEQ ID NO:57
c15-6-3.seq	+		SEQ ID NO:58
c15-7-1.seq	-		SEQ ID NO:59
c15-8-5.seq	-		SEQ ID NO:60
c17-3-1.seq	+		SEQ ID NO:61
c17-3-5.seq	+		SEQ ID NO:62
c17-5-5.seq	+		SEQ ID NO:63
c17-5-8.seq	-		SEQ ID NO:64
c17-6-2.seq	+		SEQ ID NO:65
c18-1-2.seq	+	+	DNAJ protein-like *Arabidopsis thaliana* (emb|CAB86070) SEQ ID NO:66
c18-2-1.seq	+		CCT (chaperonin containing TCP-1) b subunit *Oxytricha nova* (gb|AF188130) SEQ ID NO:67
c19-2-11.seq	+		SEQ ID NO:68
c19-3-10.seq	+		SEQ ID NO:69
c19-4-19.seq	+		SEQ ID NO:70
c19-4-22.seq	+		SEQ ID NO:71
c19-5-1.seq	-		SEQ ID NO:72
c19-5-4.seq	-		SEQ ID NO:73
c19-6-3.seq	+		SEQ ID NO:74
c19-7-4.seq	+		putative translation initiation factor 2B beta subunit (NIFb) EIF2B beta homolog *Nicotiana tabacum*

Clone number	DD+/-	N+/-/=	homology E<10-3 with at least 20%amino acids or 50% nucleic acids identical nonredundant DNA and protein sequence databases (blastx/blastn)
			(gb|AF137288) SEQ ID NO:75
c2-1-10.seq	-		SEQ ID NO:76
c2-11-14.seq	+		SEQ ID NO:77
c2-11-2.seq	+		SEQ ID NO:78
c2-2-1.seq	+		SEQ ID NO:79
c2-2-3.seq	+		SEQ ID NO:80
c2-4-1.seq	+		SEQ ID NO:81
c2-5-6.seq	+		SEQ ID NO:82
c2-6-5.seq	-		SEQ ID NO:83
c2-7-1.seq	+		nonsucrose-inducible patatin precursor -strand *Solanum brevidens* (gb|U09331) SEQ ID NO:84
c2-9-14.seq	-		SEQ ID NO:85
c20-1-4.seq	+		DNA- binding protein (pabf) *Nicotiana tabacum* (gb|U06712) SEQ ID NO:86
c3-2-4.seq	+		SEQ ID NO:87
c3-3-6.seq	+		SEQ ID NO:88
c3-4-1.seq	-		SEQ ID NO:89
c4-1-2.seq	+		SEQ ID NO:90
c4-3-3.seq	+		SEQ ID NO:91
c5-1-2.seq	+		SEQ ID NO:92
c6-8-13.seq	+		SEQ ID NO:93
c6-8-4.seq	+		SEQ ID NO:94

Clone number	DD+/-	N+/-/=	homology E<10-3 with at least 20%amino acids or 50% nucleic acids identical nonredundant DNA and protein sequence databases (blastx/blastn)
c6-8-9.seq	+		SEQ ID NO:95
c7-1-2.seq	-		SEQ ID NO:96
c7-1-6.seq	-		SEQ ID NO:97
c7-3-10.seq	-		SEQ ID NO:98
c7-3-3.seq	-		hypothetical protein *Arabidopsis thaliana* (emb|CAB62623) SEQ ID NO:99
c7-3-9.seq	-		SEQ ID NO:100
c8-1-5.seq	+		SEQ ID NO:101
c9-1-4.seq	+		hypothetical protein *Arabidopsis thaliana* (dbj|BAB08809) SEQ ID NO:102
g10-1-1.seq	+		putative ABA-responsive protein *Arabidopsis thaliana* (dbj|BAB11190) SEQ ID NO:103
g12-1-21.seq	-		hypothetical protein *Arabidopsis thaliana* (pir|T01731) SEQ ID NO:104
g12-1-5.seq	-		Putative membrane-related protein *Arabidopsis thaliana* (gb|AAD38248) SEQ ID NO:105
g14-2-4.seq	+	+	vetispiradiene synthase *Solanum tuberosum* (gb|AAD02223) SEQ ID NO:106
g14-3-10.seq	+		SEQ ID NO:107
g14-3-22.seq	+		hypothetical protein *Spinacia oleracea* (pir|T09217) SEQ ID NO:108
g14-3-3.seq	+		Sequence 162 from Patent EP0953640 *Nicotiana tabacum* (emb|AX014606) SEQ ID NO:109
g14-3-4.seq	+		HR associated Ca2+-binding protein *Phaseolus vulgaris* (gb|AAD47213) SEQ ID NO:110
g14-3-7.seq	+		SEQ ID NO:111
g15-1-37.seq	+		putative golgi transport complex protein *Arabidopsis thaliana* (gb|AAF16568) SEQ ID NO:112
g15-2-2.seq	+	=	ubiquitin *Nicotiana tabacum* (gb|U66264) able to induce HR-like lesions SEQ ID NO:113
g15-3-11.seq	-		Sequence 7 from Patent EP0953640 *Nicotiana tabacum* (emb|AX014451) SEQ ID NO:114

Clone number	DD+/-	N+/-/=	homology E<10-3 with at least 20%amino acids or 50% nucleic acids identical nonredundant DNA and protein sequence databases (blastx/blastn)
g15-3-7.seq	-		SEQ ID NO:115
g15-4-1.seq	+		SEQ ID NO:116
g17-2-13.seq	+	+	WRKY DNA binding protein *Solanum tuberosum* (emb|CAB97004) SEQ ID NO:117
g17-3-2.seq	+		SEQ ID NO:118
g18-4-7.seq	+		putative ribosomal protein L18 (60S) *Arabidopsis thaliana* (gb|AAF26138) SEQ ID NO:119
g18-5-1.seq	-		SEQ ID NO:120
g18-5-12.seq	-		SEQ ID NO:121
g18-6-12.seq	+		SEQ ID NO:122
g18-6-5.seq	+		SEQ ID NO:123
g18-7-5.seq	+		SEQ ID NO:124
g18-8-7.seq	+		SEQ ID NO:125
g19-1-5.seq	-		unknown protein *Arabidopsis thaliana* (gb|AAF23197) SEQ ID NO:126
g19-1-6.seq	+		SEQ ID NO:127
g19-1-7.seq	+		putative protein *Arabidopsis thaliana* (emb|CAB82697) SEQ ID NO:128
g19-2-1.seq	+		SEQ ID NO:129
g19-2-9.seq	+		SEQ ID NO:130
g2-1-2.seq	+	+	5-epi-aristolochene synthase *Nicotiana tabacum* (emb|Y08847) SEQ ID NO:131
g20-2-20.seq	+		hypothetical protein *Arabidopsis thaliana* (gb|AAF14679) SEQ ID NO:132
g20-2-29.seq	+		SEQ ID NO:133
g20-2-31.seq	+		SEQ ID NO:134

Clone number	DD+/-	N+/-/=	homology E<10-3 with at least 20%amino acids or 50% nucleic acids identical nonredundant DNA and protein sequence databases (blastx/blastn)
g3-1-1.seq	+		ankyrin-like protein *Arabidopsis thaliana* (dbj|BAB10271) SEQ ID NO:135
g3-1-4.seq	+	=	ADP-ribosylation factor *Capsicum annuum* (gb|AAF65512) SEQ ID NO:136
g6-2-13.seq	+	+	leucoanthocyanidin dioxygenase 2, putative; 51024-52213 *Arabidopsis thaliana* (gb|AAG21532) SEQ ID NO:137
g6-3-7.seq	+	+	ATP citrate lyase *Arabidopsis thaliana* (dbj|BAB09916) SEQ ID NO:138
g6-4-4.seq	+		SEQ ID NO:139
g6-4-5.seq	+		ATP-dependent protease proteolytic subunit ClpP-like protein *Arabidopsis thaliana* (dbj|BAB09831) SEQ ID NO:140
g7-1-1.seq	+		RNA-binding protein MEI2 (meiotic regulator), putative; 36123-32976 *Arabidopsis thaliana* (gb|AAG12640) SEQ ID NO:141
g7-1-4.seq	+		SEQ ID NO:142
g9-2-2.seq	+	+	P-glycoprotein-like protein *Arabidopsis thaliana* (emb|CAB71875) SEQ ID NO:143
g9-2-6.seq	+		SEQ ID NO:144
g9-3-17.seq	+		SEQ ID NO:145
g9-3-4.seq	+		SEQ ID NO:146
g9-5-5.seq	+		SEQ ID NO:147
g9-6-1.seq	+	+	lipoxygenase *Solanum tuberosum* (gb|AAD09202) SEQ ID NO:148
t12-1-7.seq	+	+	serine/threonine/tyrosine-specific protein kinase APK1A *Arabidopsis thaliana* (sp|Q06548) SEQ ID NO:149
t12-2-1.seq	+		chitinase class 4 *Vigna unguiculata* (pir|S57476) SEQ ID NO:150
t12-2-18.seq	+		SEQ ID NO:151

Clone number	DD+/-	N+/-/=	homology E<10-3 with at least 20%amino acids or 50% nucleic acids identical nonredundant DNA and protein sequence databases (blastx/blastn)
t18-2-5.seq	+	+	basic PRB-1b *Nicotiana tabacum* (emb|X66942) SEQ ID NO:152
t18-3-2.seq	+		SEQ ID NO:153
t18-3-6.seq	+		RNA- or ssDNA-binding protein *Vicia faba* (pir|T12196) SEQ ID NO:154
t18-4-18.seq	-		ADP-glucose pyrophosphorylase small subunit *Solanum tuberosum* (emb|X55650) SEQ ID NO:155
t-2-1-1.seq	+		ubiquitin carrier protein *Lycopersicon esculentum* (sp|P35135) SEQ ID NO:156
t2-1-3.seq	+		Hypothetical protein chlP *Nicotiana tabacum* (sp|P12204) SEQ ID NO:157
t2-6-3.seq	+		SEQ ID NO:158
t7-1-12.seq	+	=	Hypothetical protein *Arabidopsis thaliana* (gb|AAF26468) SEQ ID NO:159
t7-1-14.seq	+		t7-2-4.seq + intron SEQ ID NO:167
t7-2-4.seq	+	+	Multifunctional protein of glyoxysomal fatty acid beta-oxidation *Brassica napus* (emb|AJ000886) SEQ ID NO:161
t7-4-7.seq	+		putative glutathione S-transferase; 80986-80207 *Arabidopsis thaliana* (gb|AAF15930) SEQ ID NO:161
t7-4-8.seq	+		SEQ ID NO:162
t7-5-4.seq	+		SEQ ID NO:163
t7-5-5.seq	+		SEQ ID NO:164
t7-6-4.seq	+		SEQ ID NO:165

DD+ = induced on differential display gel

DD- = repressed on differential display gel

N+ = induced on Northern

N- = repressed on Northern

N= = constant on Northern

All GenBank, or other databases, references cited herein are hereby incorporated by reference to the same extent as if each reference were individually and specifically indicated to be incorporated by reference and were set forth in its entirety herein.

[0054] **Table 2**: Genes isolated by differential display with induction confirmed by Northern Analysis.

[0055] Columns refer, respectively, to the clone number, the name of the predicted gene, the length of isolated cDNA including both primers, the length of deduced partial protein sequence, the (putative) homologue with highest e-value identified in the database, accession number of a (putative) homologue, percentage of the amino acid sequence identity (superscript indicates homology of the same segment to similar domains localized upstream [(1)] and downstream [(2)] in the homologous protein), and the length of the high-scoring segment pair(s) identified by blastx homology search.

Clone Number	cDNA/gene name	cDNA length (bp)	Peptide length (aa)	(Putative) homologue	Accession Number	% sequence identity (aa)	HSPS length (aa)
T18-2-5	PRB-1b	448	48	pathogenesis-related protein 1b, PRB-1b (*Nicotiana tabacum*) SEQ ID NO:152	emb|X66942	100%	47
C15-1-4	CBP20	508	84	pathogen- and wound-inducible antifungal protein CBP20 (clone *cbp*20-52) (*Nicotiana tabacum*) SEQ ID NO:52	gb|AAB29959	98%	84
G2-1-2	EAS	228	8	5-epi-aristolochene synthase (clone *str*319) (*Nicotiana tabacum*) SEQ ID NO:131	emb|Y08847	100%	7
G14-2-4	VS	382	66	vetispiradiene synthase (*Solanum tuberosum*) SEQ ID NO:106	gb|AAD02223	100%	65

Clone Number	cDNA/gene name	cDNA length (bp)	Peptide length (aa)	(Putative) homologue	Accession Number	% sequence identity (aa)	HSPS length (aa)
G6-3-7	ATPC-L	397	49	ATP citrate-lyase (*Arabidopsis thaliana*) SEQ ID NO:138	dbj|BAB09916	97%	48
C18-1-2	DNAJ	397	89	DnaJ-like protein (*Arabidopsis thaliana*) SEQ ID NO:66	emb|CAB86070	75%	88
G9-2-2	MDR	505	96	P-glycoprotein-like protein (*Arabidopsis thaliana*), nucleotide binding fold NBF2 SEQ ID NO:143	emb|CAB71875	68% (1) 91% (2)	91 95
G6-2-13	DIOX	525	96	Leucoanthocyanidin dioxygenase 2-like protein (*Arabidopsis thaliana*) SEQ ID NO:137	gb|AAG21532	80%	92
G9-6-1	Lox2	269	19	Lipoxygenase (*Solanum tuberosum*) SEQ ID NO:148	gb|AAD09202	100%	17
T7-2-4	MFP	413	55	multifunctional protein of glyoxysomal fatty acid beta-oxidation (*Brassica napus*) SEQ ID NO:160	emb|AJ000886	61%	46
T12-1-7	TPK	361	75	protein tyrosine-serine-threonine kinase APK1A (*Arabidopsis thaliana*) SEQ ID NO:149	sp|Q06548	36%	82
G17-2-13	WRKY11	548	87	WRKY DNA binding protein (*Solanum tuberosum*) SEQ ID NO:117; SEQ ID NO:168; and SEQ ID NO:169	emb|CAB97004	94%	86

All GenBank, or other databases, references cited herein are hereby incorporated by reference to the same extent as if each reference were individually and specifically indicated to be incorporated by reference and were set forth in its entirety herein.

Example IV: MV-induced genes are regulated differently during the treatment

[0056] Of the antioxidant genes tested, only expression of *Gpx* and *SodCc* correlated with enhanced tolerance of pretreated samples (FIG. 3). To further investigate the transcriptional response of genes induced during adaptation to MV, Northern hybridizations were performed for a subset of identified genes (Table 2) during the pretreatment and the treatment (FIG. 4). The earliest gene induction could be observed already after one hour of the pretreatment for *MFP* and *Lox2* and is likely related to the wounding of the tissue during the leaf disc preparation. Lipoxygenase (Lox) and multifunctional protein (MFP) are both implicated in a pathway leading to lipid breakdown products such as jasmonic acid, and wounding may induce their expression (Mueller, 1997). This induction was transient and was seen in both water reference samples and MV-pretreated samples.

[0057] During the first four hours of the pretreatment, there was no discernible induction of gene expression by MV, while during the treatment, the induction was already visible after three hours. The concentration of MV during the treatment was ten times higher, suggesting that the timing of induction is concentration-dependent. All genes, except *DIOX*, were induced after 12 hours of the pretreatment with 0.1 μM MV, but more detailed time course analysis would be required to determine exact timing of induction. The low level of induction at this time point probably reflects the preceding dark period of eight hours with no photosynthetic activity. The primary site of action of MV in photosynthesizing plants are the chloroplasts (Halliwell and Gutteridge, 1989) and active photosynthesis is required for maximal generation of superoxide by this redox-cycling compound. This is in agreement with the further and much stronger induction of the mRNA level on the light during the last five hours of the pretreatment.

[0058] Expression of all genes, except *DIOX*, was further induced during the treatment with 1μM MV and the induction started within the first three hours of the treatment. In the course of the treatment, two different expression patterns were essentially recognized.

[0059] For one group of genes (*PRB-1b, CBP20, VS, MDR, DNAJ* and *WRKY11*), expression was induced by a 1 μM MV treatment in both the 0.1 μM MV-pretreated samples and water reference samples such that the level of transcript remained higher in the 0.1 μM MV-pretreated samples for at least six hours, which is the time when the difference in tolerance

between pretreated and nonpretreated samples began to be manifested. The increase in transcript levels with time was rather slow, reaching the maximum between six and nine hours in water reference samples, while it was generally three hours earlier in MV-pretreated samples. Towards the end of the treatment, the transcript level declined. A similar expression pattern was observed for antioxidant genes *GPx* and *SodCc* (FIG. 3).

[0060] The second group of genes (*EAS, TPK, Lox2* and *MFP*) was also transcriptionally induced by a 1 μM MV treatment (except *Lox2* in MV-pretreated samples) but with different kinetics. The induction was much stronger in the water reference samples, so the differences in mRNA level between MV-pretreated and the water reference samples diminished. The response was also faster, with transcript levels reaching a maximum within three hours (six hours for *MFP*) in both water reference and MV-pretreated samples. The kinetics of *ATPC-L* expression had rather intermediate character with respect to the expression patterns of the two described gene groups. Together, these data indicate the presence of at least two different mechanisms for activation of defense genes by MV.

Example V: Overexpression of *WRKY11* provokes oxidative stress tolerance

[0061] Full-length cDNA sequence was obtained by 5′ RACE using total leaf RNA and a gene-specific 3′ primer.

[0062] The corresponding gene was designated *WRKY11* (SEQ ID NOS:168 and 169) because ten nonidentical tobacco WRKY genes were already present in the database.

[0063] WRKY proteins are divided into three classes based on type and number of WRKY domains. WRKY family members show only little homology among each other outside of the WRKY domains (Eulgem, Rushton *et al.*, 2000). Database search (blastx on nrprot) revealed only one protein that is significantly similar to WRKY11 (SEQ ID NO:169) within the N-terminal part of the protein: *St*WRKY1 from potato (Dellagi, Heilbronn *et al.*, 2000).

[0064] Segregating populations (F2) of *A. thaliana* plants (C 24) transformed with *WRKY11* under control of the 35S promoter (35S-WRKY11) or with *WRKY11* fused to the *VP16* activation domain under control of the 35S promoter (35S-WRKY11-VP16) were grown on MS media with kanamycine. Approximately three-week-old seedlings resistant to kanamycine from 3:1 segregating lines (WV4 and WV9 *WRKY11-VP16* transformed lines) were transferred to the

solid media containing ½ MS salts, 1% sucrose and 2 μM methyl viologen (MV) or on plates without MV. As control plants, untransformed *A. thaliana* plants were used (C24). After three to four weeks, phenotypic differences were assessed.

[0065] On control plates without MV, no difference in growth between *WRKY11-VP16* transformants and controls were observed (FIG. 5A). On plates containing MV, growth of all plants was retarded; however, differences in growth and MV tolerance between lines overexpressing *WRKY11* and control plants were observed.

[0066] Line WV4 was more tolerant to MV than untransformed *Arabidopsis* control (C24). However, line WV9 did not differ significantly from control in its growth and MV tolerance (FIG. 5B).

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